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**Gloves contamination during endodontic treatment is one of the
sources of nosocomial endodontic *Propionibacterium acnes* infections**

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study.

Microbial load of gloves increased from beginning to end of treatment session; gloves should be changed after gaining access to the pulp space and after taking intra-operative peripaical radiograph to prevent nosocomial infections of opportunistic bacteria like *Propionibacterium acnes*.

Abstract

Introduction

The opportunistic *Propionibacterium acnes* recovered frequently from failed endodontic treatments might be the result of nosocomial endodontic infections. The study was aimed to determine if gloves worn by dentists could be one of the sources of these nosocomial infections and to investigate the *P. acnes* phlotypes involved.

Methods

The cultivable microbiota of gloves (n=8) at 4 time-points (T1-immediately after wearing gloves, T2-after access-cavity preparation, T3-after taking a working-length/master-cone radiograph, T4-before sealing the cavity) were identified using 16S rRNA gene sequencing. *recA* gene sequencing of *P. acnes* isolates was done. The phylogenetic relationship were determined using MEGA 6 (http://www.megasoftware.net/fixed_bugs.html). Data distributions were compared using Fisher's exact test; means were compared using Mann-Whitney U test in SPSSPC.

Results

The quantitative viable counts at T4 [(aerobically (2.93±0.57), anaerobically (3.35±0.43)] were greater (P<0.001) than at T1 [(aerobically (0.48±0.73), anaerobically (0.66±0.86)] and T2 [(aerobically (1.80±0.54), anaerobically (2.41±0.71)]. 80 cultivable bacterial taxa (5 phyla) were identified. The most prevalent ones were *P. acnes* and *Staphylococcus epidermidis* (100%). *recA* gene sequencing (n=88) revealed 2 phylogenetic lineages with type I split into type IA and type IB. Type II was prevalent on gloves.

Conclusion

Contamination of the gloves was detected at the final stages of the treatment. *P.*

1 *acnes* and *S. epidermidis* are the prevalent taxa on gloves and are opportunistic
2 endodontic pathogens. Changing gloves frequently, after gaining access into the
3 pulp space and also after taking the working length/master gutta-percha point
4 radiographs, is likely to reduce the risk of root canal re-infection.
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11 Keywords: *Propionibacterium acnes*, gloves, nosocomial infection
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Introduction

Propionibacterium acnes (*P. acnes*) is an anaerobic-aerotolerant Gram-positive bacillus that is a commensal bacteria on human skin (1), oral cavity, large intestine, conjunctiva (2) and external ear canal (1, 3). Numerous studies have reported *P. acnes* as an opportunistic pathogen associated with infections and inflammatory conditions (4-7). The *P. acnes* infections are also linked with surgery including brain abscesses (8), osteomyelitis after lumbar puncture (9), spondylodiscitis following epidural catheterization (10), discitis after surgery (11), post-operative mediastinitis (12), endophthalmitis (13) and endocarditis (14). Recently, *P. acnes* is emerging as a well recognized opportunistic pathogen causing various types of medical implant biofilm infection including intraocular implants (15), breast implants (16), neurosurgical shunts like ventroperitoneal and ventroatrial shunts (17), cardiovascular devices like prosthetic heart valve (18), internal fracture fixation devices, spinal hardware (19) and late prosthetic joint infections (20, 21).

Studies in dentistry have also identified *P. acnes* in parotid, periodontal and dental infections (21, 22). *P. acnes* has been identified in studies on the endodontic microbiota (23, 24) but its importance as a pathogenic has largely been ignored, because of its presence on the skin and the consequent likelihood of sample contamination. Recent studies demonstrated that *P. acnes* recovered from primary and refractory endodontic infections is an opportunistic pathogen rather than a contaminant and might be the result of nosocomial infections occurring at the time of root canal treatment (25, 26).

P. acnes has been classified into four distinct evolutionary lineages by sequence

analysis of a non-ribosomal housekeeping gene (*recA*): type IA, IB, II and III, which display differences in inflammatory properties, production of virulence determinants and association with various conditions (21, 27, 28). Previous studies demonstrated that the type II and III were associated with infections of implanted prosthesis (27, 29) and are predominant phylotypes from refractory endodontic lesions (25); whereas type IA and IB that are usually recovered from skin (25, 30) were isolated from primary endodontic cases with “open” communication with the oral cavity (26).

During endodontic treatment, maintaining sterility as much as possible is crucial. Microorganisms can contaminate the root canal if there is insufficient cross infection control leading to root canal failure (25, 31-33). To reduce contamination from the patient to practitioner and vice versa, universal precautions focused on barrier use have been developed (34). The Decontamination: Health Technical Memorandum 01-05: Decontamination in primary care dental practices 2013 version (<https://www.gov.uk/government/publications/decontamination-in-primary-care-dental-practices>) promotes the use of gloves, masks, protective eyewear, and gowns. The infection control regimens suggested by the ESE 2006 (35) and endodontic textbooks (36, 37) focus on hand washing technique, wearing of Personal Protective Equipment (PPE) to be changed between patients and use of barrier techniques within the surgery. Clean non-sterile boxed gloves are one of the key factors involved in the cross infection control during the endodontic treatment. Studies have shown that gloves can get contaminated before (38, 39) and also after use in clinical dentistry, thus being potential source for microbial contamination of the operative field (34, 40, 41). Contamination of the gloves by patient’s saliva or skin or by bacteria present in the surgery can cause the inoculation of these bacteria into

1 the root canal during the treatment (41). So far little information is available regarding
2 microbial contamination of gloves during different stages of endodontic treatment
3 session and their role in causing nosocomial endodontic infections. The objective of
4 this study was 2-fold; first to determine the potential source of nosocomial
5 endodontic *P. acnes* infections by investigating the microbiota of the pair of gloves
6 worn by the dentists at 4 different time points during endodontic treatment session
7 and second to investigate the phylotypes of *P. acnes* if isolated among the
8 microbiota present on the gloves.
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Material and Methods

Gloves Sampling

Gloves samples were collected from 8 postgraduate endodontic students performing endodontic treatment on patients in the Endodontic Department [REDACTED]

[REDACTED] The clinical
operatory surfaces were disinfected prior to treatment using Clinell universal wipes
(GAMA healthcare Ltd., London, UK). The surfaces were separated into clean and
dirty zones, and cling film barriers applied to the X-ray equipment including the
collimator and exposure button, the operating microscope, the dental unit, and dental
chair. Barrier sleeves (Henry Schein, Melville, USA) were placed over the 3 in 1
syringe, and hand pieces. The gloves used in this study were commercially available
Sempercare® Nitrile Gloves (Sempermed®, Semperit Industrial Products,
Birmingham, UK) packaged in boxes of 100 units. The postgraduate dentists were
instructed to wear a single pair of gloves for the entire treatment session of a single
patient. The dentist used Cutan® hand wash (deb, U.K.) following the 6 steps hand
washing technique recommended by Guy's and St Thomas' NHS foundation trust
([http://www.guysandstthomas.nhs.uk/patients-and-visitors/infection/washing-your-](http://www.guysandstthomas.nhs.uk/patients-and-visitors/infection/washing-your-hands.aspx#na)
[hands.aspx#na](http://www.guysandstthomas.nhs.uk/patients-and-visitors/infection/washing-your-hands.aspx#na)). The soap was lathered and rubbed on palms, backs of hands,
between fingers, fingertips, thumbs and wrists and nails for 15 seconds up to 1
minute. Hands were rinsed under clean running water and taps turned off without
touching them directly. Hands were dried with clean disposable paper towels. The
procedure was to comply with the WHO guidelines on hand hygiene in healthcare
2009 (http://apps.who.int/iris/bitstream/10665/44102/1/9789241597906_eng.pdf).
Gloves were worn immediately after drying the hands.

The samples were collected using a sterile cotton swabs rubbed all around gloves

worn on both hands at 4 time points during the treatment session; T1- immediately after wearing the gloves at the beginning of the treatment before touching anything with the gloved hands, T2- after access cavity preparation, T3- after taking a working length/master cone radiograph and T4- before sealing of the cavity prior to rubber dam removal (T4). The swabs were suspended into 1ml PRAS medium (Oxyrase, Mansfield, OH, USA) and immediately transported on ice to the laboratory.

Sterile swabs were also collected as negative control from the unhandled gloves taken immediately out from the glove boxes with the help of sterile tweezers. The swabs were plated on to the Fastidious Anaerobe Agar (FAA) supplemented with 5% [v/v] horse blood (Lab M, UK) and incubated anaerobically (in MACS-MG-1000-anaerobic workstation) for 7 days and aerobically for 3 days at 37°C.

Microbial analysis of samples

Each sample (T1-T4) was dispersed by vortexing with glass beads, serially diluted in Fastidious Anaerobe Broth (Lab M, UK) and plated onto non-selective media; duplicate plates of FAA supplemented with 5% [v/v] horse blood (Lab M, UK). The FAA plates were incubated anaerobically for 7 days and aerobically for 3 days at 37°C. After incubation colonies were counted, and predetermined number of colonies (anaerobically n = 20/time point, aerobically n = 20/time point, maximum 160/glove) were randomly selected for Gram-staining and molecular identification. The protocol used for selecting and picking the colonies was consistent with the one described in the previous studies (25, 26).

Identification of isolates

All randomly selected isolates were sub-cultured on FAA plates and grown for 24 h. Bacterial genomic DNA was extracted by boiling 100 µl of a suspension of the cultured cells prepared in sterile dH₂O for 10 min, followed by cooling on ice for 10 min and centrifuged at 13,000 × g for 2 min (21). The supernatant containing the genomic DNA was stored at -20°C prior to analysis. DNA amplification of a partial fragment of the 16S rRNA gene of the isolates was performed using universal primers; 9F (5'-3' GAGTTTGATCCTGGCTCA) and 907R (5'-3' CGTCAATTCCTTTGAGTT) (42). The amplification was carried out with the following reaction mixture (final volume, 25 µl): 0.5 µl of 9F forward primers (concentration 10 pmol/µl; MWG, United Kingdom), 0.5 µl of 907R reverse primer (concentration 10 pmol/µl; MWG), 23 µl of Reddymix buffer (Thermo Scientific, United Kingdom) and 1 µl DNA extract. The thermal cycling conditions included initial denaturation at 94°C for 10 min, denaturation at 94°C for 30 s, annealing at 49°C for 30 s and extension at 72°C for 90 s, repeated for 34 cycles and final extension at 72°C for 5 min. The amplified products were run on a 0.5% agarose gel (Sigma, United Kingdom) and visualized under UV transillumination. PCR products were cleaned for the sequence reaction with Microclean (Sigma, United Kingdom) according to the manufacturer instructions.

Amplicon sequencing was performed by using an ABI Prism BigDye Terminator sequencing kit (Applied Biosystems) with 30 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 2 min. Sequencing reaction products were run on an ABI 3730xl sequencer (Applied Biosystems). All DNA sequences were analyzed, trimmed, and aligned using BioEdit software (version

7.0.0; <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). The partial gene sequences were identified by a BLAST search of the NCBI database (<http://0-www.ncbi.nlm.nih.gov.ilsprod.ilb.neu.edu/BLAST/>), the Human Oral Microbiome database (<http://www.homd.org/>), or the Ribosome Database Project database (<http://rdp.cme.msu.edu/>). Phylogenetic trees were constructed by the neighbor-joining (NJ) method, based on 16S rRNA gene sequence comparisons, using the MEGA (version 6) program (<http://www.megasoftware.net/>).

Phylotyping of *P. acnes* isolates

Gloves *P. acnes* isolates identified by partial 16S rRNA gene sequencing were typed by partial *recA* gene sequencing (21). All selected *P. acnes* isolates were subcultured on FAA and grown for 24 h.

The *P. acnes recA* gene was amplified using primer PAR-1 (positions –96 to –75; 5'-AGCTCGGTGGGGTTCTCTCATC-3') and primer PAR-2 (positions +1105 to +1083; 5'-GCTTCCTCATACCACTGGTCATC-3'), which generated a 1,201-bp amplicon (21). The reaction mix (final volume, 25 µl) comprised of 0.5 µl of PAR-1 (concentration 10 pmol/µl; Sigma), 0.5 µl of PAR-2 (concentration 10 pmol/µl; Sigma), 23 µl of Reddymix (Thermo Scientific) and 1 µl DNA extract.

The thermal cycling conditions included initial denaturation at 95°C for 3 min, denaturation at 95°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 90 s, repeated for 35 cycles, and a final extension at 72°C for 10 min. Amplified products were run on a 0.5% agarose gel and visualized under UV transillumination.

Sequencing was performed as described above. The *P. acnes recA* sequences were

1 compared with GenBank sequences AY642055 (type IA), EU687255 (type IB),
2 AY642061 (type II), and DQ672252 (type III). NJ trees were constructed using the
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5 Jukes-Cantor method with MEGA (version 6) software (www.megasoftware.net/).
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10 11 **Statistical analysis**

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13 Single Factor: ANOVA was used to compare the quantitative viable counts between
14 the time points in SPSSPC (Version 21. IBM, USA). Data distributions were
15 compared using Fisher's exact test in the PAST-Paleontological Statistics program
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17 (version 3.06).
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Results

Quantitative Viable Counts from gloves at 4 time points (T1-T4)

No organisms were recovered from samples taken from unhandled gloves (negative control). The aerobic and anaerobic quantitative viable counts as \log_{10} Colony Forming Units (CFU) increased from the beginning (T1) to the end of the treatment session (T4) (Table 1). Few bacteria were detected at the beginning of the treatment at T1, and the microbial load was significantly less ($P < 0.001$) than that at T2, T3 and T4. The quantitative viable counts at the end of the session (T4) [(aerobically (2.93 ± 0.57) and anaerobically (3.35 ± 0.43))] were significantly greater ($P < 0.001$) than at the beginning (T1) [(aerobically (0.48 ± 0.73) and anaerobically (0.66 ± 0.86))] and before taking the radiograph (T2) [(aerobically (1.80 ± 0.54) and anaerobically (2.41 ± 0.71))]. Both aerobic and anaerobic viable counts increased from T2 to T3. At T2-aerobic counts were (1.80 ± 0.54) and increased to (2.44 ± 0.41) at T3; Similarly at T2-anaerobic counts were (2.41 ± 0.71) and increased to (2.86 ± 0.68) at T3. Moreover, the aerobic viable counts after taking the radiograph (T3) were significantly greater ($P < 0.05$) than those before taking the radiograph (T2) (Table 1).

Cultivable taxa from gloves

In the 8 glove samples using 16S rRNA sequencing 80 cultivable bacterial taxa were detected amongst the 776 isolates recovered (Table 2). The mean number of taxa from the gloves samples was 20.5 ± 4.9 .

Microbiota was dominated by the Gram-positive facultative anaerobic organisms,

1 which accounted for 60 of the 80 identified taxa. Moreover, only 2 Gram-positive
2 obligate anaerobes including *Atopobium parvulum* and *Olsenella uli* were identified;
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4 whereas 8 Gram-negative obligate anaerobes including *Alloprevotella tannerae*, 4
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6 species of *Veillonella* (*V. dispar*, *V. parvula*, *V. atypical* and *V. rogosae*), 2 species of
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8 *Prevotella* (*P. intermedia* and *P. melaninogenica*) and *Fusobacterium periodonticum*
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10 were isolated. Two Gram-negative obligate aerobes (*Delftia acidovorans* and
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12 *Kocuria sp. Oral taxon 189 Clone AW006*) were also present in the samples (Table
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24 The most prevalent bacterial taxa isolated were *P. acnes* and *Staphylococcus*
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26 *epidermidis* from all 8 gloves samples (100%) (Table 2, Fig. 1). Other
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28 Propionibacteria isolated from gloves included *P. avidum* and *Propionibacterium sp.*
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30 *Oral Taxon193 Strain Met-C3*. The mean proportion of *P. acnes* recovered from the
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32 gloves samples was 17.6% (range, 9.5% to 37.9%). Staphylococci including *S.*
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34 *epidermidis*, *S. caprae* and *S. warneri* were isolated from the gloves with *S.*
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36 *epidermidis* being the most prevalent one (Table 2, Fig. 1). The mean proportion of
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38 *S. epidermidis* recovered from the gloves samples was 18.2% (range, 8.3% to
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40 60.2%). Furthermore, *S. caprae* was present in 87.50% cases, *Kocuria sp. Oral*
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42 *taxon 189 Clone AW006* and *Streptococcus mitis/oralis* was recovered from 75%
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44 cases; followed by *Corynebacterium urealyticum* and *S. infantis* recovered from
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46 62.05%. *Actinomyces naeslundii*, *A. odontolyticus*, *S. warneri*, *S. australis*, *S.*
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48 *gordonii*, *S. mitis* bv 2 were present on 50% of the samples (Fig. 1).
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59 **Phylotyping of *P. acnes* isolates recovered from gloves**

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Partial *recA* sequences were obtained for 88 *P. acnes* isolates and on the basis of sequence alignment, 2 distinct phylogenetic lineages, type I and type II were identified. Type II was the predominant phylotype (n = 37). The Type I isolates segregated into two distinct groups, into which the sequences from known type IA (n = 27) and type IB (n = 24) sequences clustered (Fig. 1).

Comparison of the number of samples of gloves, primary endodontic infections (26) and refractory endodontic infections (25) yielding the different *P. acnes recA* phylotypes demonstrated that the number of primary endodontic samples (26) with *P. acnes* type II (1/8) is significantly different ($p < 0.05$) from both that of glove samples (6/8) and refractory endodontic samples (25). These were the only significant differences between the distribution of the *P. acnes* phylotypes among the sample types (Fig. 4).

Richness of bacterial taxa recovered from gloves

The bacterial taxa identified from the 8 gloves samples belonged to 5 phyla including *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Proteobacteria* and *Fusobacteria* (Fig. 2). The *Firmicutes* was the phylum with the largest number of bacterial taxa, including a variety of 24 different species of Streptococci, 4 Veillonella species, 3 Gemella species, 3 Staphylococcus species, 2 Granulicatella species, in addition to *Bacillus anthracis*, *Paenibacillus* sp. Oral Taxon 786 Strain F0064, *Parvimonas micra* and *Peptostreptococcus stomatis* (Fig. 2).

The next biggest phylum was *Actinobacteria* with 10 different Actinomyces species, 3 Propionibacterium species, 3 Corynebacterium species, 2 Rothia species, *Kocuria*

1 *sp. Oral Taxon 189 Clone AW006, A. parvulum, O. uli, Kytococcus sedentarius* and
2 *Microbacterium sp. Oral Taxon 184 Strain A43SC* (Fig. 2).
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7 Phylum *Proteobacteria* comprised of 2 species of *Neisseria*, *Haemophilus*
8 *parainfluenza*, *Terrahaemophilus aromaticivorans*, *D. acidovorans*, *Moraxella*
9 *osloensis*, *Agrobacterium tumefaciens*, *Rhodobacter capsulatus*, *Brevundimonas*
10 *diminuta*. Phylum *Bacteroidetes* comprised of 2 species of *Prevotella*,
11 *Capnocytophaga sp. Oral Taxon 336 Clone X089, Alloprevotella tannerae*.
12 Moreover, *Fusobacterium periodonticum* belonging to phylum *Fusobacteria* was also
13 identified in the samples (Fig. 2).
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26 Discussion

27 The main aim of the endodontic treatment is the removal of bacteria and their by-
28 products from the root canal space (43). During endodontic treatments an aseptic
29 clinical protocol is essential since bacterial contamination has been shown to
30 contribute to endodontic failure (32). This study demonstrated that the microbial load
31 of gloves worn increased greatly from the beginning to the end of the session. Since
32 the boxed examination gloves, Sempercare® Nitrile Gloves (Sempermed®, Semperit
33 Industrial Products, Birmingham, United Kingdom) are clean but non-sterile therefore
34 a few bacteria were isolated from these gloves at T1 when they were immediately
35 worn before the initiation of patient treatment. Studies have shown that gloves can
36 be contaminated before starting treatment (38, 39). The bacterial counts at T2 after
37 access cavity preparation were significantly greater than at T1. Luckey *et al.* (2006)
38 showed 10-fold increase in cultivable counts of the gloves after rubber dam
39 placement when compared to freshly worn gloves suggesting that patient contact
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1 through rubber dam placement contaminated gloves significantly (34). In this study
2 the counts after taking the radiograph (T3) and at the end of the treatment session
3 (T4) were significantly greater than those before taking the radiograph (T2). This
4 emphasizes that the bacterial contamination of these gloves might be from either
5 patient's saliva or skin; or from the bacteria present in the environment/surgery that
6 can be picked during the treatment procedure. Moreover, various studies on the
7 integrity of surgical and examination gloves have showed that all gloves gradually
8 lose their barrier integrity during use (44, 45). This discourages the prolonged use of
9 the gloves to avoid cross-infection between the dentist and the patient. Therefore, we
10 must improve our cross infection control procedures by introducing the protocol of
11 changing the gloves frequently during the endodontic treatment whenever there is
12 potential contamination like this study demonstrated that is after gaining access into
13 the pulp space and also after taking the working length/master gutta-percha point
14 radiographs.

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36 Eighty cultivable bacterial taxa belonging to five phyla were recovered from the
37 gloves samples included in the study. The prevalent taxa found on these gloves
38 included *P. acnes* and *S. epidermidis* (100%); *S. caprae* (87.50%); *Kocuria sp.* Oral
39 *taxon 189 Clone AW006* and *S. mitis/oralis* (75%); *C. urealyticum* and *S. infantis*
40 (62.05%); *A. naeslundii*, *A. odontolyticus*, *S. warneri*, *S. australis*, *S. gordonii*, *S.*
41 *mitis bv 2* (50%). Furthermore, 24 different types of Streptococci and 10 types of
42 Actinomyces were identified. Interestingly, while comparing with datasets from
43 previous studies on the endodontic microbiota, majority of bacteria recovered from
44 these gloves were consistent with the microbiota recovered from different types of
45 endodontic infections (24-26). This huge diversity of bacteria on the gloves, which
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are either picked from patient contact or from the environment, can be the source of nosocomial endodontic infections. The root canal of teeth presenting with irreversible pulpitis and no pre-operative apical radiolucency are unlikely to harbor high number of bacteria and are associated with a higher endodontic treatments success rate as observed in periapical radiographs (46). However, Patel *et al.* (2012), using reconstructed Cone Beam Computed Tomography (CBCT) images showed that the endodontic treatment of teeth with no pre-operative radiolucency revealed more failures than expected thus depicting the likelihood of bacterial introduction into the root canal space during the endodontic treatment itself despite the use of rubber dam, sterile instruments and the adherence to a strict aseptic protocol (31, 33).

Among the taxa identified from gloves, *P. acnes* along with *S. epidermidis* that is already ranked first in the nosocomial medical implant infections (47, 48), were the predominant ones. Recently, *P. acnes* is emerging as an opportunistic bacteria related to various types of medical implant biofilm infection delete (15-21, 29, 49, 50). This pathogen is considered to be responsible for the chronic or persistent low-grade implant infections, which typically manifested 3 to 36 months or longer after implantation without positive cultures, in which this pathogen is probably under recognized and underestimated (49). The reason for low grade nature of these *P. acnes* infections is its low virulence, and also encoding for the genes involved in phase variation (51), an adaptation strategy to evade immune responses and eventual degradation. In *P. acnes* low-grade infection, the inflammatory biomarkers, such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and white blood count (WBC) values are within the normal range and therefore unreliable diagnostic markers (52). Previous studies on the microbiota of endodontic infections

1 have identified *P. acnes* and coagulase-negative staphylococci, including *S.*
2 *epidermidis* among the microflora of endodontic infections (23-25). These
3
4 opportunistic pathogens, isolated from refractory lesion are likely to be the result of
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6 nosocomial infections occurring at the time of the root canal treatment and are
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8 associated with failures (25). Interestingly, Gomes *et al.* (2005) showed that sterile
9
10 gutta percha (GP) cones manipulated by gloves worn during endodontic treatment
11
12 resulted in 100% of the cones becoming contaminated, with *S. epidermidis* isolated
13
14 from 93.3% and *P. acnes* from 33.3%. Based on these findings they suggested GP
15
16 should be manipulated with sterilized tweezers and sterilized with sodium
17
18 hypochlorite prior to obturation (41).
19
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27 The genotypic analysis of the gloves *P. acnes* isolates were undertaken to assign
28
29 these to various phlotypes, so as to compare it with the previous findings where
30
31 type IA and IB are mostly associated with skin (25, 30) and II and III primarily
32
33 recovered from implant infections (21, 29) and refractory endodontic infections (25).
34
35
36

37
38 Phylogenetic analysis showed that type II was the prevalent phlotypes found on the
39
40 gloves. Moreover, no significant difference was demonstrated between the number
41
42 of samples of gloves and refractory endodontic infections (25) yielding *P. acnes* type
43
44 II. Therefore this study also revealed a potential source of *P. acnes* type II that
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48
49 can lead to nosocomial refractory endodontic infections.
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54 **Acknowledgments**

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56
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58
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The authors deny any conflicts of interest related to this study.

Table 1 Quantitative viable counts of the gloves at 4 time points during endodontic treatment; T1- immediately after wearing the gloves at the beginning of the treatment, T2- after access cavity preparation, T3- after taking a working length/master cone radiograph and T4- before sealing of the cavity prior to rubber dam removal (T4).

Microbial counts as log ₁₀ per sample							
T1 Mean ± S E		T2 Mean ± S E		T3 Mean ± S E		T4 Mean ± S E	
Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
0.48 (0.73)	0.66 (0.86)	1.80 (0.54) *	2.41 (0.71) *	2.44 (0.41) *** **	2.86 (0.68) ***	2.93 (0.57) *****	3.35 (0.43) *****

*****Values at T4 significantly greater than values at T1 and T2 (P < 0.001).

****Values at T4 significantly greater than values at T3 (P < 0.05).

***Values at T3 significantly greater than T1 (P < 0.01).

** Values at T3 significantly greater than values at T2 (P < 0.05).

*Values at T2 significantly greater than values at T1 (P < 0.001).

Table 2: Distribution of all 80 cultivable bacterial taxa identified among 776 isolates recovered from gloves.

Organism	Presence on Gloves							
	1 (23)	2 (12)	3 (15)	4 (25)	5 (24)	6 (19)	7 (20)	8 (26)
Gram-positive								
Obligate anaerobes								
<i>Atopobium parvulum</i>	–	–	–	–	–	–	–	+
<i>Olsenella uli</i>	–	–	–	–	+	–	–	–
Facultative anaerobes								
<i>Actinomyces graevenitzi</i>	–	–	–	–	–	–	–	+
<i>Actinomyces naeslundii</i>	+	+	–	–	+	–	+	–
<i>Actinomyces odontolyticus</i>	+	–	–	+	+	–	+	–
<i>Actinomyces</i> sp. Oral Taxon 169 clone AG004	+	+	–	–	+	–	+	–
<i>Actinomyces</i> sp. Oral Taxon 170 clone AP064	–	–	–	–	–	–	–	+
<i>Actinomyces</i> sp. Oral Taxon 171 clone JA063	+	–	–	–	–	–	–	–
<i>Actinomyces</i> sp. Oral Taxon 172 clone CT047	+	–	–	–	+	–	–	–
<i>Actinomyces</i> sp. Oral Taxon 180 strain C3M24	+	–	–	–	+	–	–	+
<i>Actinomyces</i> sp. Oral Taxon 180 strain C29KA	–	–	–	–	–	+	–	–
<i>Actinomyces</i> sp. Oral Taxon 180 strain Hal-1083	–	–	–	–	–	+	–	+
<i>Arsenicococcus</i> sp. Oral Taxon 190 Strain B46KS	–	–	–	+	+	–	–	–
<i>Bacillus anthracis</i>	+	–	–	–	–	–	–	–
<i>Corynebacterium diphtheriae</i>	–	–	–	+	–	+	–	–
<i>Corynebacterium mucifaciens</i>	+	–	+	–	+	–	–	–
<i>Corynebacterium</i> sp. Oral Taxon 184 Strain A43SC	–	–	+	–	–	–	–	–
<i>Corynebacterium urealyticum</i>	–	–	+	+	+	+	+	–
<i>Gemella haemolysans</i>	+	–	–	–	–	–	–	–
<i>Gemella morbillorum</i>	–	–	–	–	–	–	–	+
<i>Gemella sanguinis</i>	+	–	–	–	–	–	–	–

1	<i>Granulicatella elegans</i>	-	-	-	-	-	+	-	-
2	<i>Granulicatella adiacens</i>	-	-	-	-	-	-	-	+
3									
4	<i>Kytococcus sedentarius</i>	-	-	-	-	+	-	-	-
5	<i>Microbacterium</i> sp. Oral Taxon186								
6	Strain C24KA	-	-	-	-	+	-	-	-
7	<i>Paenibacillus</i> sp. Oral Taxon 786								
8	Strain F0064	-	-	+	-	-	-	-	-
9									
10	<i>Parvimonas micra</i>	-	-	-	-	+	-	-	-
11									
12	<i>Peptostreptococcus stomatis</i>	-	-	-	-	-	-	+	-
13	<i>Propionibacterium acnes</i>	+	+	+	+	+	+	+	+
14									
15	<i>Propionibacterium avidum</i>	-	-	+	-	-	-	-	-
16	<i>Propionibacterium</i> sp. Oral Taxon193								
17	Strain Met-C3	-	-	-	-	+	-	-	-
18									
19	<i>Rothia dentocariosa</i>	-	-	-	+	-	-	-	-
20									
21	<i>Rothia mucilaginosa</i>	-	-	-	-	+	-	-	-
22									
23	<i>Staphylococcus caprae</i>	+	+	-	+	+	+	+	+
24	<i>Staphylococcus epidermidis</i>	+	+	+	+	+	+	+	+
25									
26	<i>Staphylococcus warneri</i>	-	+	-	-	-	+	+	+
27									
28	<i>Streptococcus anginosus</i>	-	-	-	-	-	-	-	+
29									
30	<i>Streptococcus australis</i>	-	-	-	+	+	+	+	-
31	<i>Streptococcus constellatus</i>	+	-	-	-	-	-	-	+
32									
33	<i>Streptococcus gordonii</i>	-	-	+	+	-	+	-	+
34									
35	<i>Streptococcus cristatus</i>	+	-	+	-	-	-	+	-
36	<i>Streptococcus infantis</i>	+	-	+	+	+	-	+	-
37									
38	<i>Streptococcus mitis/oralis</i>	+	+	+	+	-	-	+	+
39									
40	<i>Streptococcus mitis</i> bv 2	+	-	+	-	-	-	+	+
41	<i>Streptococcus parasanguinis</i>	+	-	-	+	+	+	-	-
42									
43	<i>Streptococcus peroris</i>	-	+	-	-	-	-	-	-
44									
45	<i>Streptococcus salivarius</i>	+	-	-	+	-	-	-	+
46									
47	<i>Streptococcus sanguinis</i>	-	+	-	-	+	-	-	-
48	<i>Streptococcus</i> sp. Oral Taxon 061								
49	clone DP009	+	-	-	-	-	-	-	-
50	<i>Streptococcus</i> sp. Oral Taxon 061								
51	Clone DN025	-	-	-	+	-	+	-	+
52	<i>Streptococcus</i> sp. Oral taxon 064								
53	Clone C5MLM037	-	-	-	+	-	-	-	-
54	<i>Streptococcus</i> sp. Oral Taxon 066								
55	Clone FN051	-	-	+	+	-	-	-	+
56	<i>Streptococcus</i> sp. Oral Taxon 184								
57	Clone T4-E3	-	-	+	-	-	-	-	-
58	<i>Streptococcus</i> sp. Oral Taxon 431								
59	Clone C4AKM023	-	-	-	-	+	-	-	-
60									
61									
62									
63									
64									
65									

<i>Streptococcus</i> sp. Oral Taxon 158 Clone BM035	-	-	-	-	-	-	+	-
<i>Streptococcus</i> sp. Oral Taxon 071 Clone P4PA-13	-	-	-	-	-	-	+	-
<i>Streptococcus</i> sp. Oral Taxon 071 Clone C3ALM006	-	-	-	-	-	-	-	+
<i>Streptococcus</i> sp. Oral Taxon 065 Clone FN042	-	-	-	-	-	-	-	+
<i>Streptococcus</i> sp. Oral Taxon 431 Clone C4AKM023	-	-	-	-	-	-	-	+
<i>Streptococcus vestibularis</i>	-	-	-	+	-	-	-	-

Gram-negative

Obligate aerobes

<i>Delftia acidovorans</i>	-	-	-	-	+	-	-	-
<i>Kocuria</i> sp. Oral taxon 189 Clone AW006	-	+	+	-	+	+	+	+

Obligate anaerobes

<i>Alloprevotella tanneriae</i>	-	-	-	-	+	-	-	-
<i>Fusobacterium periodonticum</i>	-	-	-	-	-	-	+	-
<i>Prevotella intermedia</i>	-	-	-	-	-	-	+	-
<i>Prevotella melaninogenica</i>	-	-	-	-	-	+	-	-
<i>Veillonella dispar</i>	-	+	-	-	-	+	-	-
<i>Veillonella parvula</i>	-	+	-	-	-	-	-	-
<i>Veillonella atypica</i>	-	-	-	-	-	+	-	-
<i>Veillonella rogosae</i>	-	-	-	-	-	+	+	-

Facultative anaerobes

<i>Agrobacterium tumefaciens</i>	-	-	-	+	-	-	-	-
<i>Brevundimonas diminuta</i>	-	-	-	+	-	-	-	-
<i>Capnocytophaga</i> sp. Oral Taxon 336 Clone X089	-	-	-	-	+	-	-	-
<i>Haemophilus parainfluenza</i>	+	-	-	+	-	-	-	+
<i>Moraxella osloensis</i>	-	-	-	+	-	-	-	-
<i>Mycoplasma faucium</i>	-	-	-	+	-	-	-	-
<i>Neisseria flavescens</i>	-	-	-	+	-	-	-	+
<i>Neisseria mucosa</i>	-	-	-	-	-	-	-	+
<i>Rhodobacter capsulatus</i>	-	-	-	-	-	+	-	-
<i>Terrahaemophilus aromaticus</i>	+	-	-	+	-	-	-	-

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Figure legends

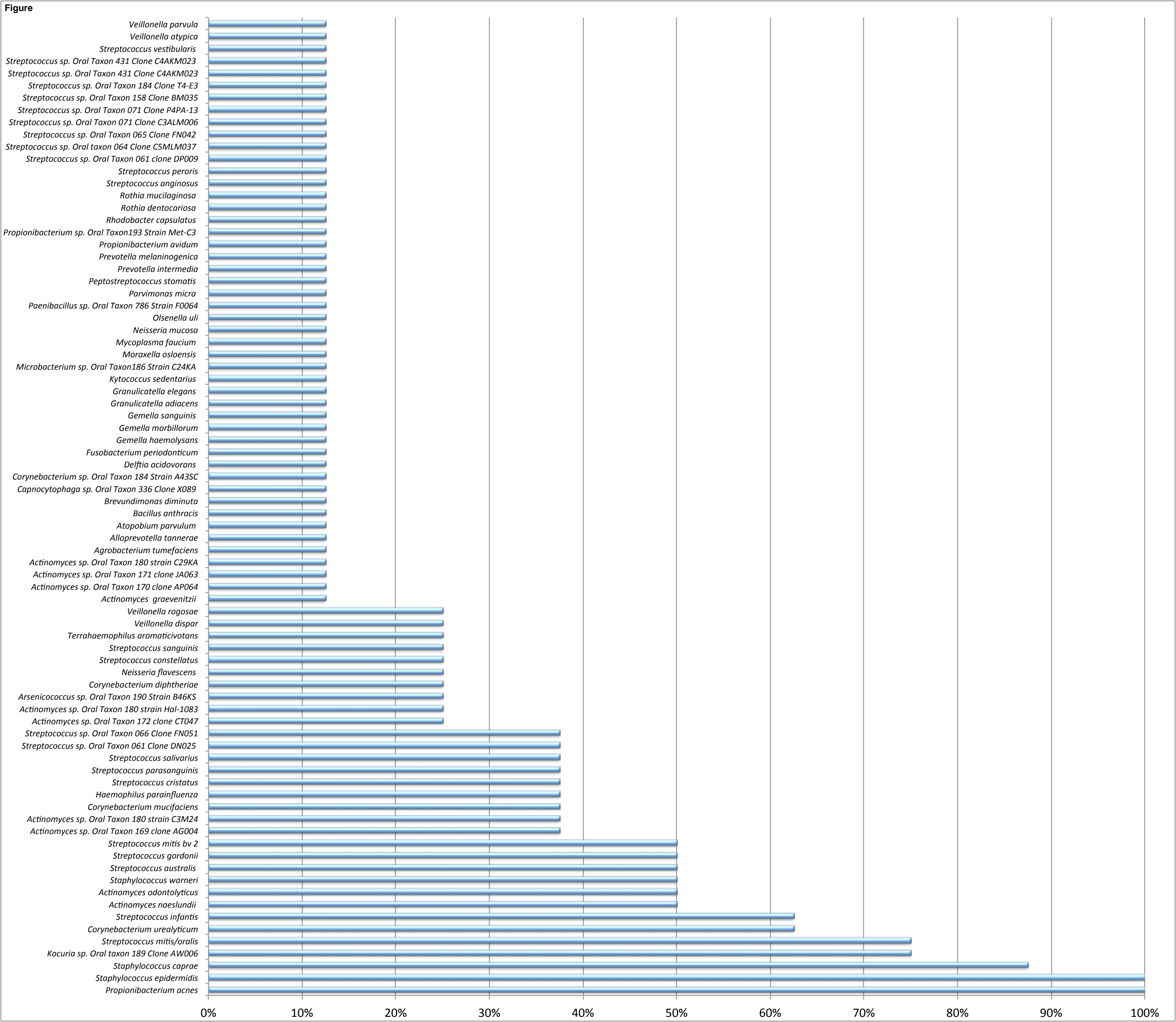
Fig. 1 Prevalence of the 80 microbial taxa identified from 8 gloves samples.

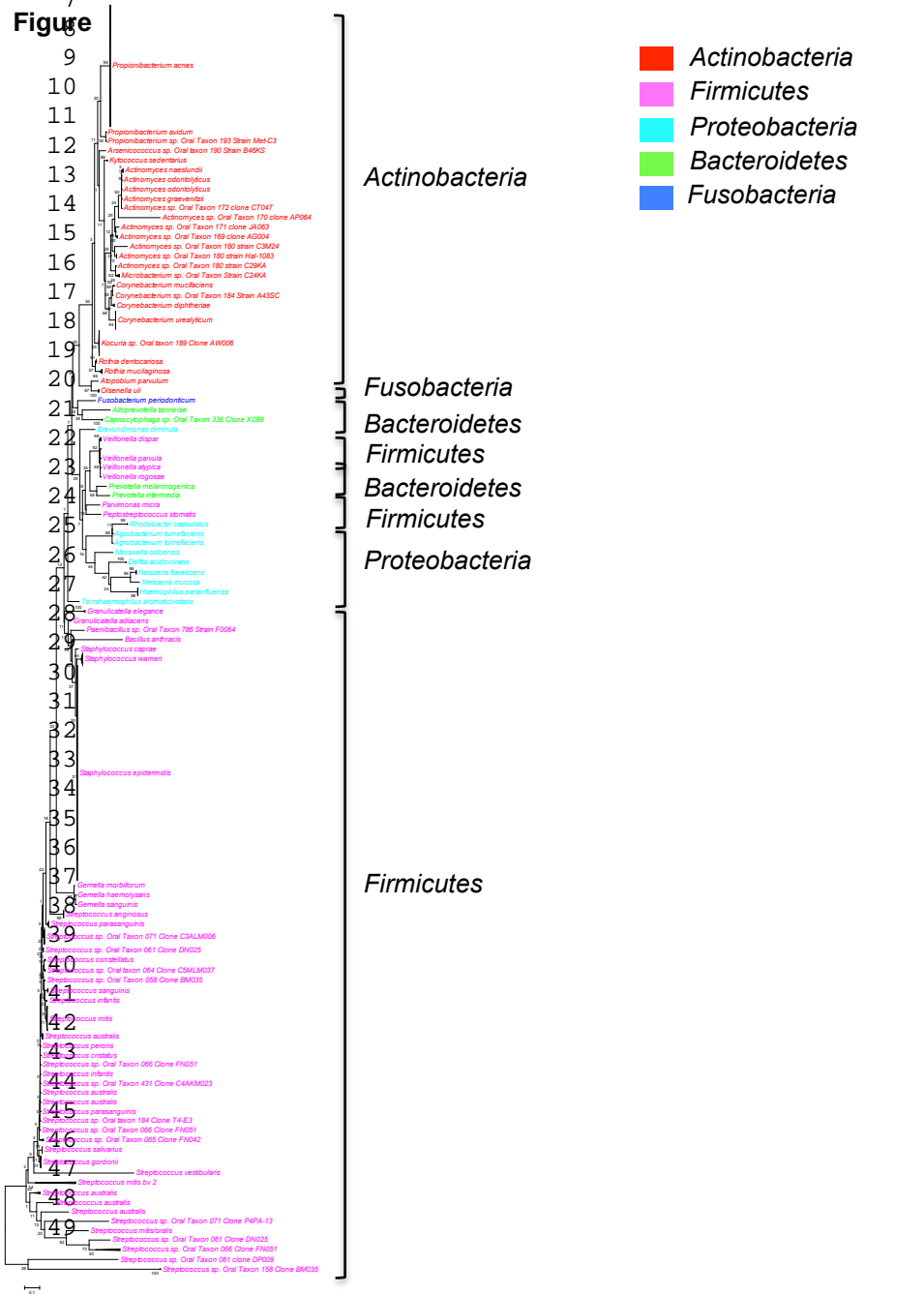
Fig. 2 Phylogenetic tree showing all 80 bacterial taxa belonging to 5 phyla from 776 isolates identified from the 7 gloves samples. The tree was constructed by the neighbor-joining method based on 16S rRNA gene sequence comparisons. The scale bar represents 0.05 substitutions per nucleotide position. The numbers at the node of the tree indicate bootstrap values for each node out of 500 bootstrap resampling.

Fig. 3 Phylogenetic tree obtained by partial *recA* sequencing of 88 endodontic *P. acnes* isolates showing three distinct *P. acnes* phylotypes Type IA, IB and II. Neighbor-joining (NJ) tree was constructed using the Jukes-Cantor method with MEGA (version 6). *P. acnes recA* sequences were compared with GenBank sequences [AY642055](#) (type IA), [EU687255](#) (type IB), [AY642061](#) (type II), and [DQ672252](#) (type III). The scale bar represents 0.05 substitutions per nucleotide position. The numbers at the node of the tree indicate bootstrap values for each node out of 500 bootstrap resampling.

Fig. 4 Comparison of the number of samples of gloves (n=8), primary endodontic infections (n=8) (26) and refractory endodontic infections (n=17) (25) yielding the different *P. acnes recA* phylotypes. Fisher's exact test was used for analysis.

***The number of primary endodontic samples (26) with *P. acnes* type II (1/8) is significantly different ($p < 0.05$) from both that of glove samples (6/8) and refractory endodontic samples (25). These were the only significant differences between the distribution of the *P. acnes* phylotypes among the sample types.





Figure

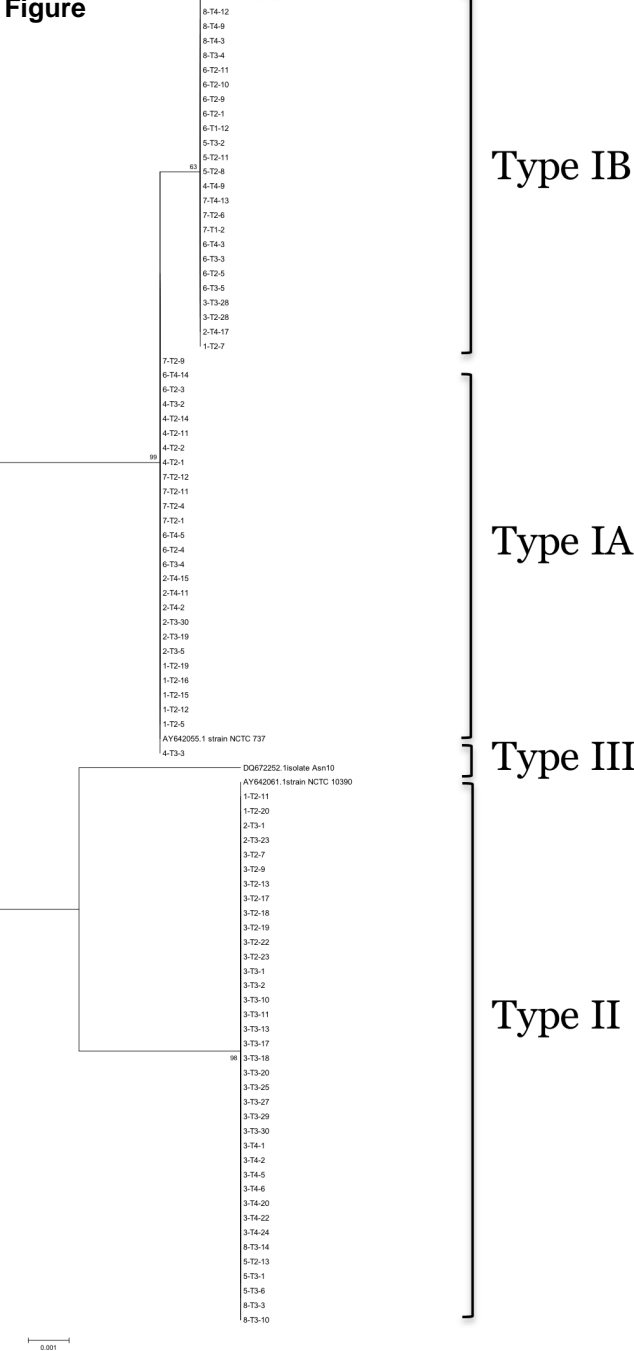
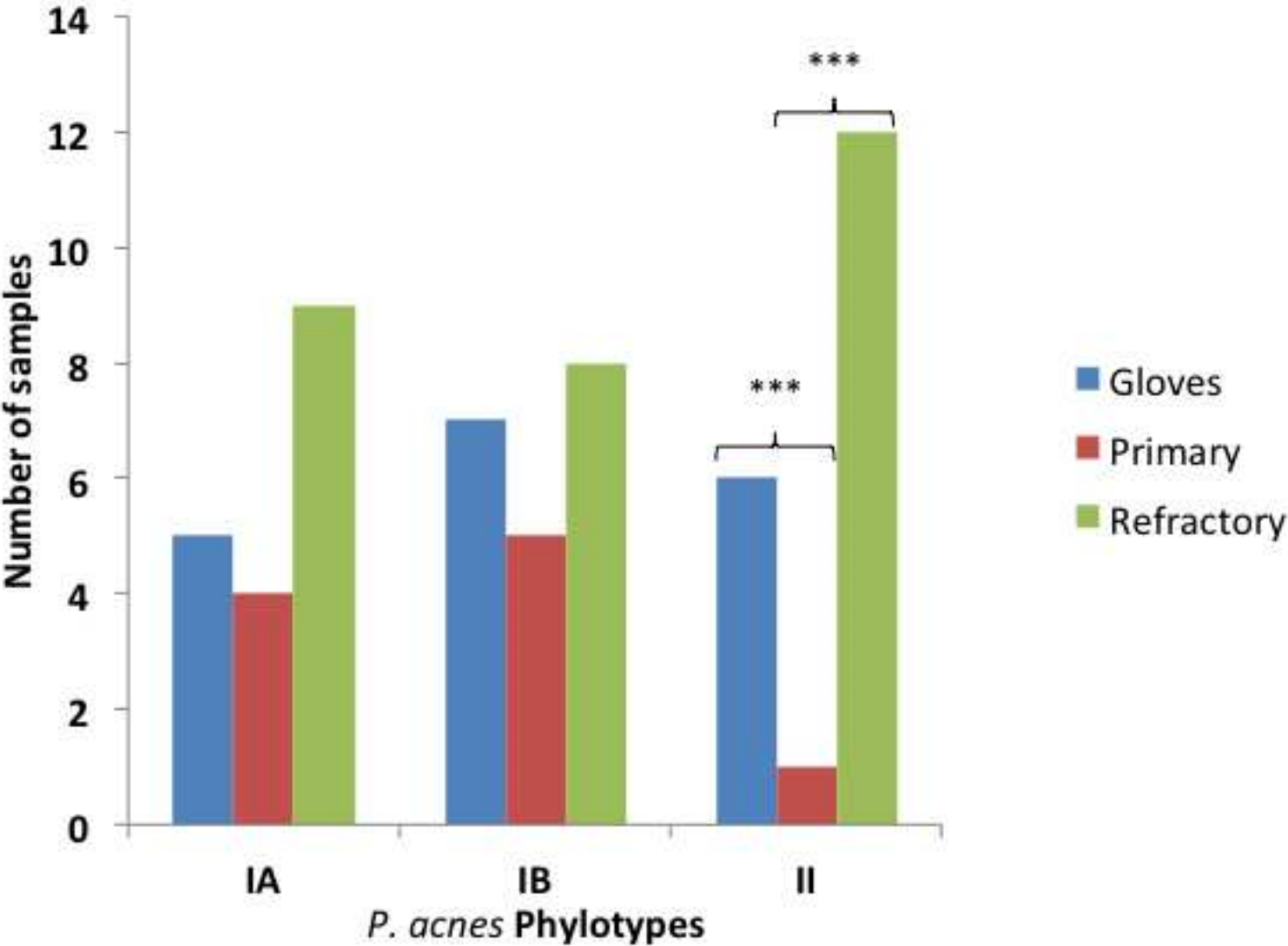


Figure
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Associate editor comments:

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2
3
4 1) A major concern is the absence of baseline readings, or negative controls,
5 being samples from unhandled gloves immediately out of the box. The T1
6 samples were taken after the gloves had been handled. Please provide these
7 baseline data or at the very least justify this omission in the discussion.
8
9

10
11 **Answer: We appreciate the comments of the Associate Editor. This study**
12 **was carried out to investigate the microbial load of the gloves during**
13 **endodontic use. At this stage it is difficult to repeat the entire experiment**
14 **to include the baseline CFU's. However as suggested by the editor and**
15 **reviewer, we have conducted an experiment to include the negative**
16 **control. The negative control samples were taken from eight unhandled**
17 **gloves with the help of sterile tweezers taken immediately out from**
18 **different glove boxes placed in different clinical surgeries in the**
19 **Endodontic Department. While holding the gloves with the sterile**
20 **tweezers, samples were taken with the sterile swab and plated**
21 **immediately on to the Fastidious Anaerobe Agar (FAA) supplemented with**
22 **5% [v/v] horse blood (Lab M, UK). The FAA plates were incubated**
23 **anaerobically (in MACS-MG-1000-anaerobic workstation) for 7 days and**
24 **aerobically for 3 days at 37°C.**
25
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29
30 **No organism was recovered from these plates incubated both aerobically**
31 **and anaerobically.**
32

33
34 **This is added in the Methods: (Page 6/line 37) and results : (Page 10/line**
35 **7)**
36

37
38 2) Details need to be included in the Methods section of the hand washing
39 protocol used by the 8 residents providing the samples.
40

41 **Answer: We appreciate comments of the Associate Editor and Hand**
42 **washing protocol is added in the Methods section. : (Page 6/line 20)**
43

- 44
45
 - What hand washing protocols were used prior to placement of gloves?
46 Product used to wash hands?
47

48 **Answer: The dentist used Cutan® hand wash (deb, U.K.)**
49 **(www.debskincare.co.uk) following the 6 steps hand washing technique**
50 **recommended by Guy's and St Thomas' NHS foundation trust**
51 **([http://www.guysandstthomas.nhs.uk/patients-and-](http://www.guysandstthomas.nhs.uk/patients-and-visitors/infection/washing-your-hands.aspx#na)**
52 **visitors/infection/washing-your-hands.aspx#na).** The soap was
53 lathered and rubbed on the palms, the backs of hands, between fingers,
54 fingertips, thumbs and wrists and nails for 15 seconds up to 1 minute.
55 Hands were rinsed under clean running water and taps turned off
56 without touching them directly. Hands were dried with clean disposable
57 paper towels. The procedure was to comply with the WHO guidelines
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on hand hygiene in healthcare 2009
(http://apps.who.int/iris/bitstream/10665/44102/1/9789241597906_eng.pdf).

- How long after washing their hands did they place the gloves?

Answer: Gloves were worn immediately after drying the hands.

- Did they touch the surface ultimately sampled prior to placing the gloves on their hands?

Answer: The gloves were first worn and then the surfaces of the gloves were sampled. Therefore, there are chances that gloves surfaces that were sampled were handled by bare hands while putting them on.

- Does "immediately after wearing the gloves" mean sampling before touching anything with the gloved hands?

Answer: Yes, at T1 sampling was done immediately after wearing the gloves before touching anything with the gloved hands. This is added in the methods.

- How were the clinic operatory surfaces that would have been touched treated?

Answer: The clinical operatory surfaces were disinfected prior to treatment using Clinell universal wipes (GAMA healthcare Ltd., London, UK). The surfaces were separated into clean and dirty zones, and cling film barriers applied to the X-ray equipment including the collimator and exposure button, the operating microscope, the dental unit, and dental chair. Barrier sleeves (Henry Schein, Melville, USA) were placed over the 3 in 1 syringe, and hand pieces (added in the Methods section. : (Page 6/line 11)

- Who handled the Xray equipment – were these surfaces covered with a barrier?

Answer: The postgraduate dentist carrying out the treatment positioned the phosphor plate covered with a Soredex Opticover™ (SOREDEX, UK) within a Rinn endodontic holder (Dentsply, UK) and the x-ray tube, which was covered with a cling film barrier.

3) Figure 4 - suggest including a descriptor for X axis, such as "phylotypes".

Answer: Figure 4 has been corrected as suggested and X-axis title is added as "*P. acnes* phylotype".

Reviewer #1: The authors should be applauded for writing this noteworthy manuscript titled, "Gloves contamination during endodontic treatment is one of the sources of nosocomial endodontic *Propionibacterium acnes* infections".
Introduction:

1) "Recent studies demonstrated that *P. acnes* recovered from primary and refractory endodontic infections is an opportunistic pathogen rather than a contaminant and might be the result of nosocomial infections occurring at the time of root canal treatment (25-28)." References # 27 and #28 do not pertain and support the statement provided by the authors.

Answer: We appreciate comments of the referee and references # 27 and # 28 has been deleted and subsequent cited references have been renumbered.

2) Introduction: "Contamination of the gloves by patient's saliva or skin or by bacteria present in the surgery can cause the inoculation of these bacteria into the root canal during the treatment." Reference is needed.

Answer: Reference has been added.

3) Figure 1 is not legible

Answer: Old Figure 1 has been replaced with the new high resolution Figure 1.

4) Table 1: Why in T3 the Anaerobic microbial count was statistically not significant? Was it a technical error?

Answer: We appreciate the comment of the referee. Table 1 showed that both aerobic and anaerobic viable counts increased from T2 to T3. At T2- Aerobic counts were (1.80 ± 0.54) and increased to (2.44 ± 0.41) at T3; Similarly at T2-Anaerobic counts were (2.41 ± 0.71) and increased to (2.86 ± 0.68) at T3. Statistically significant increase was found between T2 and T3 aerobically. There is no technical error; the results showed that although there was an increase in the viable counts of both aerobic as well as anaerobic bacteria on gloves, however after taking the radiograph only the aerobic bacteria significantly increased on the gloves. This could be related to the contact of the gloved hands with the external surfaces while taking the radiograph like x-ray equipment, which is more likely to harbour aerobic than anaerobic bacteria, which are more commonly present in the oral cavity.

(Page 10/line 27) This has been added in the explanation of results.

"Both aerobic and anaerobic viable counts increased from T2 to T3. At T2-

1 aerobic counts were (1.80± 0.54) and increased to (2.44 ± 0.41)] at T3;
2 Similarly at T2-anaerobic counts were (2.41 ± 0.71) and increased to
3 (2.86± (0.68) at T3. Moreover, the aerobic viable counts after taking the
4 radiograph (T3) were significantly greater (P < 0.05) than those before
5 taking the radiograph (T2) (Table 1)."
6

7 5) Discussion: The first paragraph does not add to the paper. I would suggest
8 deleting it.
9

10
11 **Answer: (Page 13/line 20) The previous first paragraph has been modified**
12 **by deleting few lines and adding it in continuation with the previous 2nd**
13 **paragraph (now 1st paragraph).**
14
15

16 Discussion: "This study demonstrated that the microbial load of gloves worn
17 increased greatly from the beginning to the end of the session." Did the authors
18 actually demonstrate this finding? Mann-Whitney statistical analysis does not do
19 that. At T3 there was no statistically significant difference with regards to the
20 anaerobic microbial count. Microbial count at T4 was not significantly greater
21 than values at T3. Values at T3 were not significantly greater than values at T1.
22 Something happened at T3, so my guess is a technical error? The authors need to
23 offer an explanation for this in the Discussion section.
24
25
26
27

28
29 **Answer:**

- 30 • Thank you for the comments. There was a mistake in and Statistical
31 analysis on (Page 9/line 24)_has been corrected:
32

33
34 **Single Factor: ANOVA was used to compare the quantitative viable counts**
35 **between the time points.**
36

- 37 • The results of the study in Table 1 clearly showed that both aerobic
38 and anaerobic CFU's increased significantly from the beginning at T1
39 to the end of the treatment session at T4 (P < 0.001).
40
41
42 • The increase in the aerobic and anaerobic viable counts with the
43 significance difference only found in the aerobic CFU's is explained
44 while answering the above question.
45
46
47 • Table 1 has been update with more details of the statistical
48 difference and the results showed that at T4 aerobic quantitative
49 viable counts are also significantly greater than those at T3 (P <
50 0.05). Moreover Values at T3 are significantly greater than T1 (P <
51 0.001).
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56 Reviewer #2: The authors are commended for raising the awareness of microbial
57 contamination during endodontic treatment.
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1 The authors aimed to study the presence and phylotypes of *P. acnes* in non-
2 sterile dental gloves (1), and correlate the findings with their previous findings
3 that *P. acnes* isolated from refractory endodontic lesions was from patients' skin
4 and oral environment (2). By showing that *P. acnes* was progressively picked up
5 by dental gloves when handling patients during endodontic treatment, the
6 authors put forth a recommendation that non-sterile dental gloves should be
7 changed at suggested time points, so that introduction of *P. acnes* into root
8 canals could be minimized and healing outcome improved (3).
9

10
11 With regards to (1), the presence of *P. acnes* in non-sterile dental gloves and the
12 increase in microbial load as treatment progressed, the authors have shown
13 convincing results.
14

15
16 With regards to (2), that *P. acnes* was progressively picked up from handling
17 patients alone, this is not convincingly shown as there was no control gloves that
18 went through the process of treatment without handling patients. The authors
19 could not conclude that this emphasized the source of contamination (Page 14
20 Lines 10-17) unless the study is revised to include a control for the operatory
21 environment and equipment as a potential source of *P. acnes*.
22
23

24
25 **Answer: We appreciate the comments of the referee. (page 14 Line 10) has**
26 **be rewritten including other possible sources of *P. acnes* present on the**
27 **gloves.**
28

29
30 **This emphasizes that the bacterial contamination of these gloves might be**
31 **from either patient's saliva or skin; or from the bacteria present in the**
32 **environment/surgery that can be picked during the treatment procedure.**
33

34
35 While the authors might desire to challenge or provide much-needed evidence
36 for the current accepted practice of cross-infection control that includes
37 changing gloves whenever there is a breach or potential contamination, the
38 authors should not make recommendations for specific time-points for changing
39 non-sterile dental gloves (Page 14 Lines 24-34) without studying the minimum
40 quantitative viable counts that would result in refractory endodontic lesions.
41
42

43
44 **Answer: We appreciate the comments of the referee. However, in our**
45 **opinion it is bit more complicated than just minimum number of viable**
46 **counts that can result in the refractory endodontic lesions; whether or not**
47 **the refractory infection will occur or not depends other factors such as:**
48

- 49 • Bioburden left in the root canal after chemo-mechanical
50 debridment,
- 51 • Host immune response
52

53
54 **Therefore, if the conditions inside the root canal are in favor of the growth**
55 **of the bacteria any quantitative viable count above none can lead to**
56 **refractory endodontic infection.**
57

58
59 **The reason for recommending the time points for changing the gloves i.e.**
60 **gaining access into the pulp space and also after taking the working**
61
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length/master gutta-percha point radiographs is because these are the sensitive time points where the canal is more likely to be contaminated and the evidence from this study demonstrates that gloves are contaminated at these time points thus increasing the chances of nosocomial contamination.

The statement has been modified. (Page 14 Lines 24)

Therefore, we must improve our cross infection control procedures by introducing the protocol of changing the gloves frequently during the endodontic treatment whenever there is potential contamination like this study demonstrated that is after gaining access into the pulp space and also after taking the working length/master gutta-percha point radiographs.

The manuscript is generally clearly written. However, some spelling and grammatical errors are present and should be corrected before publication.

Answer: The manuscript has been rechecked for spellings and grammatical errors.